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Research Article

HLA-A Alleles Differentially Associate with Severity to *Plasmodium falciparum* Malaria Infection in Ibadan, Nigeria

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ABSTRACT

Human Leukocyte Antigen (HLA), particularly HLA-B and class II alleles have been differentially associated with disease outcomes in different populations following infection with the malaria Plasmodium falciparum. However, the effect of HLA-A on malaria infection and/or disease is not fully understood. Recently, HLA-A alleles have been suggested to play a role in the outcome of P. falciparum malaria infection in a Malian study. Herein, we investigated the association between HLA-A alleles and the outcome of malaria infection in children in Ibadan southwest Nigeria. HLA-A genotyping was performed on 393 children samples (DNA) using the sequence-based method. We compared genotype and allele frequencies data obtained from these Nigerian children; 176 with asymptomatic malaria infection (controls), 124 with uncomplicated malaria and 93 children with severe malaria (51 severe malarial anaemia and 42 cerebral malaria). We found a high frequency of HLA-A*36:01 (13.5%) in the entire studied population and also confirmed the high frequency of a previously reported allele of African origin (HLA-A*30:01). After adjusting for age and parasite density, we found a significant association between HLA-A*20:01:01 (OR = 3.19, $p < 0.001$) and susceptibility to severe malarial anaemia. We also found significant associations between HLA-A* 29:02:01 (OR = 7.26, $p = 0.008$) and A* 66:02 (Or = 4.19, $p = 0.03$) and susceptibility to cerebral malaria. Our findings suggest that HLA-A alleles play a role in the outcome of malaria in children in Ibadan. These findings may help elucidate the molecular background of malaria resistance in the study population.

Keywords: Severe malaria, HLA-A, Ibadan, children

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INTRODUCTION

Clinical presentations of falciparum malaria vary in pattern and severity (from asymptomatic infections all the way to the very severe forms), which is largely due to factors from the parasite and the genetic and internal environment of human host. Malaria is a major driving force for the selection of many genes and systems including the human leukocyte antigen (HLA) system, a highly polymorphic supergene complex with extensive diversity across different populations. More than 200 genes have been mapped to the HLA region but only 20% of them code for leukocyte antigens that are involved in regulating host immunity (Corzo et al., 1995; Klein and Sato, 2000). HLA genes directly involved in immune regulation can broadly be divided into two main classes: HLA class I (A, B, and C) and HLA class II (including HLA-DR), molecules identified for their roles in presentation of antigen to CD8 and CD4 T cells respectively. Three of the genes (HLA-A, HLA-B, and HLA-C) within the class I region are considered to be highly polymorphic (Robinson et al., 2016). HLA-B is by far the most polymorphic with 4,459 known alleles followed by HLA-A and HLA-C (3,657and 3,290 alleles, respectively, according to the latest release 3.26.0.1 of November 11th 2016).

Over the years, HLA polymorphisms have been associated with the outcome of clinical malaria in different populations (Mosaad, 2015). Hill et al. (1991) described the first association study between malaria and HLA in West Africans, demonstrating the presence of HLA-Bw53 and -DRB1*13:02 alleles with reduced susceptibility to severe malaria. Another study in Thai malaria patients showed a significant difference in the distribution of HLA-B46, -B56 and –DRB1*10:01 between non-cerebral severe malaria and severe malaria groups (Hananantachai et al., 2005). Other

studies in other populations have demonstrated different allelic distributions and associations between HLA and malaria (Shankarkumar et al., 2002; Blackwell et al., 2009).

HLA-A like the other classes of HLA has been shown to have the ability to present malarial antigens to cytotoxic T-cells during malaria infection (Lyke et al., 2004). Nonetheless, HLA-A has not attracted much attention compared with HLA-B and HLA class II alleles in malaria immunity over the years, as most studies have found HLA-B and other HLA class II alleles to be associated with malaria outcomes (Hananantachai et al., 2005; Blackwell et al., 2009; Yamazaki et al., 2011). However, Lyke et al. (2011) identified HLA-A*30:01 as a potential susceptibility factor for cerebral malaria in Malian children. This will be the first study to suggest an association between HLA-A alleles and not with the well-known HLA-B53 (B*53:01) or with the HLA class II block DRB1*13:02-DQB1*05:01 and malaria (Hill et al., 1991; Hill et al., 1992).We therefore typed the HLA-A loci in children presenting with asymptomatic, uncomplicated and severe forms of malaria in Ibadan southwest Nigeria to determine the distribution of HLA-A alleles and their association with malaria outcome.

MATERIALS AND METHODS

Study area: The study was carried out in Ibadan, a city in southwest of Nigeria, a holoendemic area for malaria. Ibadan is the capital of Oyo state and is located 3˚5´ east of the Greenwich meridian and latitude 7˚23´ north of the equator (Lawal et al., 2009). The climate is that of tropical rainforest zone, with a warm dry-season from November to April and a rainy season from May to October (Gbotosho et al., 2009). Malaria transmission in Ibadan occurs year round but is more intense during the rainy season. Children between the ages of 6 to 96 months were recruited from the Children's Emergency ward of the University College Hospital, Ibadan, St. Mary Hospital, Eleta Ibadan and the Oni Memorial Hospital, Ibadan. The study was carried out in the Institute of Child Health, University College Hospital, Ibadan and in the UK Medical Research Council (MRC), The Gambia over a four-year period (1998-2011).

Selection of human subjects: A total of 393 children were studied; 176 children with asymptomatic malaria served as the controls, while the cases consisted of 124 children with uncomplicated malaria (UM) and 93 children with severe malaria (SM) as defined by World Health Organization criteria (WHO, 2000). The children were recruited from Children's Emergency ward and Children Out-patient Clinic of the three hospitals. The inclusion criteria for the children in the asymptomatic malaria infection group were the presence of asexual forms of *P. falciparum* in peripheral blood films, an axillary temperature of <37.5ºC and the absence of the clinical symptoms of malaria. The uncomplicated malaria group comprised of clinically ill children (without signs of severity or evidence of vital organ dysfunction) with at least a temperature of greater than 37.5˚C and the presence of asexual forms of *P. falciparum* in peripheral blood film. The severe malaria group was defined as presence of *P. falciparum* asexual parasitaemia and no other obvious cause of symptoms, impaired consciousness or unrousable coma, haemoglobin < 5g/dL or haematocrit < 15%. Severe malaria was further categorized into severe malaria anaemia (defined as packed cell volume of <15%) and cerebral malaria (unrousable coma which persisted for more than 30 minutes after a convulsion, Blantyre score of ≤2). Ethical approval was obtained from the joint UI/UCH ethical review committee. Informed consent was obtained from the parents of the children recruited for the study. All recruited patients had a clinical history taken and physical examination done.

Collection of blood samples and DNA extraction : A 5ml quantity of venous blood was collected into sterile EDTA tubes on recruitment. Thick and thin blood smears were prepared to determine parasite densities. Malaria parasites were examined on 5% Giemsa-stained thick and thin blood smears stained for 20minutes. The parasites were counted against 200 white blood cells and the parasite densities were calculated based on an assumed total white blood cell of 8,000/µL (WHO, 2010). DNA was extracted from whole blood samples using the Nucleon BACC I Kit (Tepnel Life Sciences, UK) by following manufacturer's instructions with slight modifications. The DNA samples were stored at -20˚C prior to HLA genotyping. The quality and concentration of the DNA samples was tested using the Nano Drop Spectrophotometer ND – 1000.

Genotyping : HLA typing was done following the sequence-based method as described by Yindom et al (2010). Briefly, genomic DNA from each individual was amplified using locus-specific primers that flanked exons 2 and 3 of HLA-A locus. Sequence-specific primers were then used to sequence the amplified products. Each locus was amplified by polymerase chain reaction (PCR) in a 20µl reaction mixture consisting of 2 µl of 10X PCR buffer, 0.4µl of 10mM dNTPs, 0.8µl of 50mM MgCl2, 1.0 Unit of Taq polymerase (Bioline Ltd, London, UK), 0.4µl of 10µM of each primer and 150ng DNA . Amplifications were performed using a PTC-200 (tetrad) Thermal Cycler (MJ Research, Inc., MA, USA) programmed as follows: for HLA-A, 1 cycle of 96 °C for 2 minutes, 30 cycles of 96 °C for 25 seconds, 60 °C for 25 seconds, and 72 °C for 1 minute, followed by 1 cycle of 72 °C for 7 minutes and 4 °C; HLA-B, 1 cycle of 96 °C for 2 minutes, 40 cycles of 96 °C for 15 seconds, 62 °C for 15 seconds, and 72 °C for 1 minute, followed by 1 cycle of 72 °C for 7 minutes and 4 °C; and HLA-C, 1 cycle of 96 °C for 2 minutes, 30 cycles of 96 °C for 25 seconds, 70 °C for 25 seconds, and 72 °C for 1 minute, followed by 1 cycle of 72 °C for 7 minutes and 4 °C. The efficiency of the PCR reaction and the sizes of the amplified products were checked by electrophoresing 5µl of the products in 2% agarose gel and staining with ethidium bromide. Gels were visualized using a UV transilluminator and a gel picture taken with a Kodak gel image documentation camera (DC120). The remaining 15µl products were purified using AMPure kit (Agentcourt Bioscience Corporation, Beverly, USA).

Sequencing reaction and interpretation: The BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) was used to

sequence the purified products in both directions following the manufacturer's instructions. Briefly, the mix contained (per reaction) 2.07 µl of 5X buffer, 0.25 µl of BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA), 5.36 µl of water, 0.32 µl of primer, and 2.0 µl of purified PCR products. The sequencing conditions were 1 cycle of 96 °C for 1 minute; followed by 30 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes; and finally one cycle of 4 °C on hold. The sequencing products were purified using Sephadex G-50 superfine (Sigma-Aldrich, Dorset, UK) in MultiScreen HV 0.45 µm plates (Millipore Corporation, Watford, UK). Cleaned products were analysed using ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

Descriptive statistics (means, standard deviations, medians, ranges) were computed for continuous variables while frequencies were computed for categorical variables. Subjects were grouped according to clinical category: asymptomatic malaria infection, acute uncomplicated malaria and severe malaria (cerebral malaria and severe malarial anaemia). The group comparisons of categorical variables were done using the chi square (χ^2) test. *HLA-A* allele frequencies were calculated using SPSS v16 (SPSS Inc, Chicago, USA). Allele frequencies were compared between pairs of malaria groups, using a $\chi 2$ test based on a 2 X 2 contingency table. Binary logistic regression with adjustment for age and parasite density was used to test for significant associations between HLA-A alleles and clinical malaria groups. Allele frequency was calculated as a percentage of the total number of alleles in the locus of interest. Allele frequencies were compared among the clinical groups: severe malaria and uncomplicated (severe malarial anaemia *vs* uncomplicated and cerebral malaria *vs* uncomplicated); severe malaria and asymptomatic (severe malarial anaemia *vs* asymptomatic malaria infection and cerebral malaria *vs* asymptomatic malaria); uncomplicated and asymptomatic malaria..

RESULTS

The study population comprised 393 children, 198 (50.4%) were males and 195(49.6%) were females with a mean age of 51±31 months. Based on the criteria of the World Health Organization, 124 were classified as uncomplicated malaria, 176 were asymptomatic malaria and 93 were severe malaria. The severe malaria subjects included 42 cerebral malaria and 51 severe malarial anaemia patients. The different categories of subjects differed significantly in mean log parasite density and mean packed cell volume ($p<0.001$ for both clinical parameters) [Table 1]. The mean body temperature (in ˚C) of the children in the asymptomatic, uncomplicated and severe malaria groups were 36.6, 37.5 and 38.1 ($p = 0.001$) respectively. The children in the asymptomatic group were the oldest with a mean age of 58.2 months, while the children in the severe malaria group were the youngest with a mean age of 43.4 months (see Table 1).

Frequencies of HLA-A alleles in Ibadan: Thirty-five HLA-A alleles were detected in the study population. Table 2 shows the frequencies of the alleles. Of these alleles, 20 (57%) had frequencies of more than 1%. The most common allele was A*36:01 (with a frequency of 13.5%), closely followed by A*23:01 (10.5%) and A*03:01 (10.0%).

Table 1:
Characteristics according to Clinical category

Parameters	ASM	UM	SM	p value
Number of Participants	176	124	93	
Gender				
Male	87(49.4)	64 (51.6)	47 (50.5)	0.73
Female	89 (50.6)	60 (48.4)	46 (49.5)	
Age (geometric mean)	47.6	37.5	35.5	<0.001*
Mean body temperature (°C)	36.6	37.5	38.1	0.001*
Mean log parasite density	2.67	2.91	3.39	<0.001

*significant p values

Table 2:
Allele frequencies$^{\infty}$ of HLA-A

HLA-A alleles	2N	% Frequency
A*01:01	9	1.14
A*02:01	65	8.27
A*02:02	34	4.33
A*02:05	11	1.39
A*03:01	71	9.03
A*23:01	84	10.69
A*26:01	9	1.15
A*29:02	9	1.15
A*30:01	77	9.79
A*30:01	30	3.82
A*30:02	26	3.31
A*33:03	48	6.11
A*34:02	43	5.47
A*36:01	106	13.49
A*66:01	11	1.39
A*66:02	15	1.91
A*68:01	14	1.78
A*68:02	60	7.63
A*68:10	32	4.07
A*74:01	27	3.44
Total	786	100

$^{\infty}$*Only alleles with at least 1% frequency are shown in this table*

Association of HLA-A alleles with malaria

The frequency of the allele, A*66:01 was highest in the severe malaria group. Another allele, A*02:01 was also close to significance with the highest frequency found in the severe malaria group.

Further comparison between children in the asymptomatic and the severe malaria sub-groups (cerebral malaria and severe malarial anaemia) showed a significantly increased frequency of A*66:01 in both the cerebral and severe malarial anaemia groups ($p = 0.01$ and $p = 0.02$ respectively). The HLA-A alleles, A*66:02 and A*29:02 were significantly more common in the cerebral malaria group

when compared with children in the asymptomatic group (p = 0.01 and p = 0.02 respectively) (Table 3). When HLA allele frequencies were compared between the severe malaria and uncomplicated group, A*02:01 was found to be significantly higher in the severe malaria group than in the uncomplicated malaria group(p=0.02)(Table 4). Further analyses, comparing HLA-A allele frequencies between uncomplicated and severe malarial anaemia groups showed a significantly higher proportion of the same allele, A*02:01 in the severe malarial anaemia group (p < 0.001). Comparison of HLA-A frequencies between the uncomplicated malaria and cerebral malaria group showed a significant difference in the frequency of A*29:02 with the uncomplicated malaria group having the lower frequency of the allele (p = 0.01) (Table 4).

Table 3:
Comparison of HLA-A allele frequencies in Nigerian children between asymptomatic and severe malaria groups

Allele	**Group comparisons**			**Sub-group comparisons**			
	AsyM (2n=352)	**SM (2n=186)**	**Pc**	**SMA (2n=102)**	**Pc**	**CM (2n=84)**	**Pc**
A*01:01	0.011 (4)	0.016 (3)	0.64	0.02 (2)	0.52	0.012 (1)	0.97
A*02:01	0.085 (30)	0.118 (22)	0.22	**0.176 (18)**	**0.02***	0.048 (4)	0.25
A*02:02	0.04 (14)	0.065 (12)	0.20	0.059 (6)	0.41	0.071 (6)	0.21
A*03:01	0.085 (30)	0.027 (5)	0.81	0.049 (5)	0.23	0.143 (12)	0.85
A*23:01	0.085 (30)	0.091 (17)	0.85	0.098 (10)	0.69	0.06 (5)	0.44
A*30:01	0.111 (39)	0.005 (1)	0.62	0.059 (6)	0.12	0.143 (12)	0.41
A*30:02	0.034 (12)	0.097 (18)	0.91	0.02 (2)	0.46	0.048 (4)	0.55
A*34:02	0.062 (22)	0.032 (6)	0.13	0.039 (4)	0.37	0.024 (2)	0.16
A*36:01	0.139 (49)	0.043 (8)	0.61	0.127 (13)	0.76	0.119 (10)	0.63
A*66:01	**0.003 (1)**	**0.032 (6)**	**0.04***	**0.029 (3)**	**0.02***	**0.036 (3)**	**0.01***
A*66:02	**0.011 (4)**	0.124 (23)	0.08	0.01 (1)	0.89	**0.06 (5)**	**0.01***
A*29:02	**0.009 (3)**	0.032 (6)	0.09	0.01 (1)	0.90	**0.048 (4)**	**0.02***
A*68:02	0.071 (25)	0.032 (6)	0.44	0.059 (6)	0.67	0.048 (4)	0.45
A*68:01	0.014 (5)	0.027 (5)	0.74	0.039 (4)	0.98	0.024 (2)	0.53
A*74:01	0.026 (9)	0.054 (10)	0.16	0.039 (4)	0.47	0.06 (5)	0.11

pc indicates p values after Bonferonni correction for multiple comparison
**significant p values after correction with Bonferonni for multiple comparison*

Table 4:
Comparison of HLA-A allele frequencies in Nigerian children between uncomplicated and severe malaria groups

Allele	Group comparisons			Sub-group comparisons			
	UM (n=248)	**SM (2n=186)**	**Pc**	**SMA (2n=102)**	**Pc**	**CM (2n=84)**	**Pc**
A*01:01	0.008 (2)	0.016 (3)	0.45	0.02 (2)	0.36	0.012 (1)	0.75
A*02:01	**0.052 (13)**	**0.118 (22)**	0.02*	**0.176 (18)**	**0.00***	0.048 (4)	0.86
A*02:02	0.032 (8)	0.065 (12)	0.11	0.059 (6)	0.25	0.071 (6)	0.12
A*03:01	0.097 (24)	0.091 (17)	0.85	0.049 (5)	0.14	0.143 (12)	0.24
A*23:01	0.133 (33)	0.081 (15)	0.08	0.098 (15)	0.36	0.06 (5)	0.07
A*30:01	0.081 (20)	0.097 (18)	0.56	0.059 (6)	0.48	0.143 (12)	0.09
A*30:02	0.032 (8)	0.032 (6)	1.00	0.02 (2)	0.52	0.048 (4)	1.00
A*34:02	0.060 (15)	0.032 (6)	0.18	0.039 (4)	0.43	0.024 (2)	0.19

Regression models

In regression models, after adjusting for age and parasite density, only the allele A*02:01 remained associated with severe malarial anaemia with an increased risk of developing severe malarial anaemia (OR=3.19, p<0.001, 95% CI1.75-5.83). Two alleles, A*29:02 and A*66:02 were independent predictors of cerebral malaria, after adjusting for age and parasite density. A*29:02 was associated with an increased risk of developing cerebral malaria (OR=7.26, p=0.008, 95% CI 1.89-27.85), and A*66:02 with an increased risk of cerebral malaria (OR=4.19, p=0.03, 95% CI 1.32-13.37).

DISCUSSION

The HLA system is the most polymorphic of all human genetic systems. HLA-A however, appears to have the lowest level of heterozygosity in all populations (Cao et al., 2004). Several studies have shown the effect of genetic diversity on the innate human immune response but the mechanisms are still not clearly understood. Different HLA haplotypes have been shown in several studies to protect from natural infection with malaria parasites (Garamszegi, 2014).

Table 5a:
Logistic Regression Model for Potential Predictors of Cerebral Malaria

HLA Allele	Cerebral Malaria OR	95% CI	Pc
A*29:02	7.26	1.89 – 27.85	0.008*
A*66:01	3.56	0.91 – 13.86	0.07
A*66:02	4.19	1.32 – 13.37	0.03*
A*02:01	0.62	0.22 – 1.76	0.37
Age	0.99	0.98 – 0.99	0.03*
Parasite Density	1.00	1.00 – 1.00	0.08

All models included adjustment for age, gender and parasite density. Reference category: asymptomatic malaria; controlled for age and parasite density;
pc indicates p values after Bonferonni correction for multiple comparison
**significant p values after correction with Bonferonni for multiple comparison*

Table 5b:
Logistic Regression Model for Potential Predictors of Severe Malarial Anaemia

HLA Allele	Severe Malaria Anaemia OR	95% CI	p_c
A*29:02	0.98	0.12 – 7.98	0.985
A*66:01	3.11	0.79 – 12.13	0.10
A*66:02	0.21	0.01 – 3.48	0.28
A*02:01	3.19	1.75 – 5.83	0.0001*
Age	0.99	0.98 – 0.99	0.007*
Parasite Density	1.0	1.0 – 1.0	0.001*

*All models included adjustment for age, gender and parasite density. Reference category: asymptomatic malaria; controlled for age and parasite density; * P < 0.05. p_c, p values after Bonferonni correction*

HLA-B*53 and the HLA class II block DRB1*13:02-DQB1*05:01 were found to be associated with protection against severe malaria in West Africa (Hill et al., 1991; Aidoo et al., 2000), malaria associated anaemia and reinfection (May et al., 2001) and cerebral malaria (Hananantachai et al., 2005). We found the frequency of HLA-A*36:01 (13.5%) to be highest in our study population (table 2). Although there was no strong association, the frequency of A*36:01 was highest in the asymptomatic control group compared with the other malaria groups. The high prevalence of this allele in this study population of sick children perhaps suggests that A*36:01 may have a yet unidentified role in paediatric malaria and warrant further investigations. We also found a relatively high frequency of the A*30:01 allele (9.79%) in our study population in line with the result from the study carried out by Lyke et al. (2011). They described A*30:01 as one of the most common HLA alleles in populations of African descent and our data supports their findings. Lyke et al. (2011) found that A*30:01 was associated with the development of cerebral malaria among young children in Mali, but we did not find any association between A* 30:01 and malaria outcome in the present study. This disparity could be due to regional differences in genetic diversity that is characteristic of African populations and has been shown to contribute to differences in immune response to diseases or disease outcomes (Cao et al., 2004; Abdennaji et al., 2006).

HLA A*02:01 was significantly associated with severe malaria with a 3-fold increased risk of developing severe malarial anaemia but there was no such association with cerebral malaria (Table 5b). The alleles, A*29:02 and A*66:02 were independent predictors of cerebral malaria and not of severe malarial anaemia (Table 5a). A*29:02 was associated with a 7-fold increased risk and A*66:02 with a 4-fold increased risk of cerebral malaria. Severe malarial anaemia and cerebral malaria are life-threatening complications in which the mechanisms for the development and progression are generally not fully understood. These two malaria syndromes differ in clinical manifestations and pathogenesis, the suppression of erythropoiesis being suggested as a principal mechanism that distinguishes severe malarial anaemia from cerebral malaria. Studies have also shown that these two phenotypes differ in immunological pathways with different immune responses being associated with the different phenotypes; Low interleukin 10, lower levels of IL-6 and high levels of tumour necrosis factor (TNF) have been shown to have a strong association with severe malarial anaemia while thrombocytopenia, significantly elevated levels of IL-6 and IL-10, elevated tissue plasminogen activator inhibitor 1 levels had the strongest associations with cerebral malaria (Biemba, 2000; Lyke et al., 2004; Thuma et al., 2011).These distinct associations as shown in our study may reflect the different facets of the complexity of the host responses to the infection and progression of the disease.

In conclusion, we found HLA-A* 36:01 to be the most common allele in our studied population. We demonstrate (for the first time in this group of children from South western Nigeria) that HLA-A alleles may differentially modulate the severity of malaria disease following peadiatric infection with *P. falciparum* as distinct HLA-A alleles correlate with different disease outcomes: A*02:01 associates with severe malarial anaemia while A*29:02 and A*66:02 independently associate with the most severe form known as cerebral malaria. We recommend that further epidemiological as well as functional studies be conducted in the sub-region and indeed in other endemic part of the world to ascertain the contribution of specific HLA-A alleles in malaria disease outcomes.

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The authors declare that they have no conflicting interests

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